Removal of fermentation inhibitors from alkaline peroxide pretreated and enzymatically hydrolyzed wheat straw: Production of butanol from hydrolysate using *Clostridium beijerinckii* in batch reactors

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ABSTRACT

In these studies, alkaline peroxide pretreatment of wheat straw was investigated. Pretreated wheat straw was hydrolyzed using cellulolytic and xylanolytic enzymes, and the hydrolysate was used to produce butanol using *Clostridium beijerinckii* P260. The culture produced less than $2.59\,g\,L^{-1}$ acetone–butanol–ethanol (ABE) from alkaline peroxide wheat straw hydrolysate (APWSH) that had not been treated to reduce salt concentration (a neutralization product). However, fermentation was successful after inhibitors (salts) were removed from the hydrolysate by electrodialysis. A control glucose fermentation resulted in the production of $21.37\,g\,L^{-1}$ ABE, while salt removed APWSH resulted in the production of $22.17\,g\,L^{-1}$ ABE. In the two fermentations, reactor productivities were 0.30 and $0.55\,g\,L^{-1}\,h^{-1}$, respectively. A comparison of use of different substrates (corn fiber, wheat straw) and different pretreatment techniques (dilute sulfuric acid, alkaline peroxide) suggests that generation of inhibitors is substrate and pretreatment specific.

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1. Introduction

Currently, $24.96\,hm^3$ (6.50 billion gallons) of ethanol is produced in the United States from corn annually [1]. The capacity of ethanol production is $30.30\,hm^3$ (7.89 billion gallons). With another $21.27\,hm^3$ (5.54 billion gallons) of ethanol production facilities under construction, the total production capacity will be over $51.57\,hm^3$ (13.43 billion gallons) per year in the near future [1]. Further increase in ethanol production will necessitate use of agricultural residues or dedicated energy crops. The use of agricultural residues or energy crops will require hydrolysis of these

residues to simple sugars prior to fermentation as there are no cultures that can efficiently hydrolyze biomass and simultaneously ferment to ethanol or butanol (a biofuel). Hydrolysis of biomass requires physical and chemical pretreatment and hydrolysis using enzymes. During pretreatment, biomass is subjected to severe conditions such as a combination of high temperature and reaction with chemicals such as (i) dilute sulfuric acid, (ii) dilute alkali, (iii) ammonia, (iv) hot water, and (v) alkaline peroxide. As a result of pretreatment conditions, fermentation inhibitors such as salts, phenolic acids (ρ-coumaric and ferulic), and aldehydes are produced [2]. It has been observed that salts are

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potent fermentation inhibitors [3–6]. Removal of these inhibitors prior to fermentation is essential for successful biofuel fermentation.

Another problem that exists with the use of biomass hydrolysates for ethanol fermentation is inefficient uptake and metabolism of biomass component sugars such as pentoses. Production of acetone–butanol–ethanol (ABE) by *Clostridium acetobutylicum/Clostridium beijerinckii* is a process where pentoses along with hexoses are efficiently used by the cultures. Use of both sugars may make ABE fermentation more attractive than ethanol or other solvent production processes. Additionally, butanol has higher energy content than ethanol, can be used in existing pipelines, mixed with gasoline in any proportion, and is less hazardous to handle [7]. In our previous studies, we have demonstrated that ABE can be produced from dilute sulfuric acid pretreated wheat straw (WS) without any inhibition to the producing organism (*C. beijerinckii* P260) [8–10]. During those studies, it was also observed that the rate of fermentation was faster than control fermentations where glucose was used. In recent studies, it has been demonstrated that alkaline peroxide pretreatment is an effective technique to pretreat WS [11]. Hence, the objective of these studies was to produce ABE from alkaline peroxide pretreated WS hydrolysate (WSH) and compare fermentation performance of the two hydrolysates (dilute sulfuric acid and alkaline peroxide).

2. Materials and methods

2.1. Culture and inoculum development

C. beijerinckii P260 was a generous gift from Professor David Jones, University of Otago (Dunedin, New Zealand). Methods for culture maintenance and inoculum development have been documented elsewhere [9,12]. The inoculums were developed in 125 mL screw-capped bottles containing 100 mL medium as detailed previously [9].

2.2. Wheat straw (WS) pretreatment and hydrolysis

Details of WS including cultivar, harvest, and storage have been given in our previous report [10]. WS was pretreated with alkaline peroxide and hydrolyzed using enzymes. In order to treat WS, 886 g dry WS was treated with 3.33 L of 1.20 M NaOH (Sigma Chemicals). To this mixture 716 g of 30% H_2O_2 solution was added followed by raising volume to 6.66 L with distilled water. The mixture was allowed to stand for 1 h, before adding additional 3.33 L distilled water. The total volume of the mixture was 10 L. The mixture was incubated at 35 °C for 24 h followed by adjusting pH to 5.0 with approximately 250 mL concentrated HCl (Sigma Chemicals). To the reaction mixture 140 mL of each of three enzyme solutions [Celluclast 1.5 L (cellulase; Sigma Chemicals); Novozyme 188 (β-glucosidase; Sigma Chemicals); and Viscostar 150 L (xylanase; Dyadic Corporation, Jupiter, FL, USA)] were added and mixed well. Finally, the mixture was incubated at 45 °C for 72 h with agitation at 80 rpm. After incubation, the alkaline peroxide WSH (APWSH) was filtered twice (11 μm pore size, 110 mm diameter; Whatman International Ltd.,

Maid Stone, England) to remove sediments. Following this, the clear liquid was filter sterilized by passing through a 0.2 μm filter. The sterilized solution was stored in a presterilized screw-capped bottle at 4 °C for fermentation studies to be conducted later. In this solution, total sugar concentration was 44.3 g L^{-1} (glucose 24.6, xylose 16.0, arabinose 2.3, and galactose 1.4 g L^{-1}).

2.3. Fermentation studies

Fermentation studies were conducted in 50 mL screw-capped Pyrex[TM] bottles containing 30 mL medium. Prior to dispensing APWSH into bottles, medium pH was adjusted to 6.5 with 4 M KOH solution under sterile conditions. Approximately, 15.7 g L^{-1} sugar (by adding 1.18 mL of 400 g L^{-1} filter-sterilized glucose solution) was added to reach a total sugar concentration 60 g L^{-1}. To the APWSH solution, 0.75 mL of 40 g L^{-1} presterilized yeast extract (Sigma Chemicals) solution and 0.3 mL (10 mL into 1000 mL medium) of each of the three P2 medium stock solutions [13] (buffer, vitamins, and minerals) were added. Fermentation conditions and sampling procedures have been described in our earlier article [9]. The bottle was transferred to an anaerobic jar (BBL; Fisher Scientific, Pittsburgh, PA, USA) for 48 h to create anaerobic conditions in the medium. This was followed by adding 2 mL actively growing culture.

Fermentation studies, where various amounts of salt were added to the medium, were performed in 125 mL screw-capped bottles containing 100 mL medium. The purpose of these studies was to check if salt (NaCl, Sigma Chemicals) at 20–25 g L^{-1} was inhibitory to the *C. beijerinckii* P260. In order to prepare the medium 6 g of glucose, 0.1 g of yeast extract (Sigma Chemicals) and specified amount of salt (2.0–25.0 g L^{-1}) were dissolved in 100 mL water. The pH of this solution was adjusted to 6.5 using 4 M KOH prior to autoclaving at 121 °C for 15 min. Upon cooling to room temperature, the bottles were transferred to an anaerobic jar, containing BD GasPak[TM] EZ envelopes (Sigma Chemicals) with indicators, for 48 h to create anaerobic conditions. Prior to inoculation with 6–7 mL actively growing culture, 1 mL each of stock solutions (buffer, vitamin, and mineral) were added.

2.4. Salt removal

Salts were removed from APWSH by electrodialysis. The electrodialysis apparatus [high-efficiency electrodialysis (HEED), Model 07-B-10 from EET Corp., Harriman, TN, USA] contained a stack of 10 membranes of area 71 cm^2 each for a total membrane area of 710.0 cm^2. The membranes that were used included Neosepta AXE-01 (anion permeable) and Neosepta CMS (cation permeable). The electrodialysis apparatus was operated under the following conditions: fixed voltage 20 V, feed (APWSH, D tank) flow rate 3.84 L min^{-1}, concentrate (C tank, NaCl solution with initial or zero time salt concentration 17.0 g L^{-1}) flow rate 3.84 L min^{-1}, and electrolyte (E tank, 20 g L^{-1} sodium sulfate) solution with a flow rate of 1.92 L min^{-1}. The electrodialyis machine removed ions such as Na$^+$ and Cl$^-$ from APWSH (or D tank) and accumulated in NaCl solution (C tank). As a result of removing NaCl (or salts) from APWSH, the salt concentration
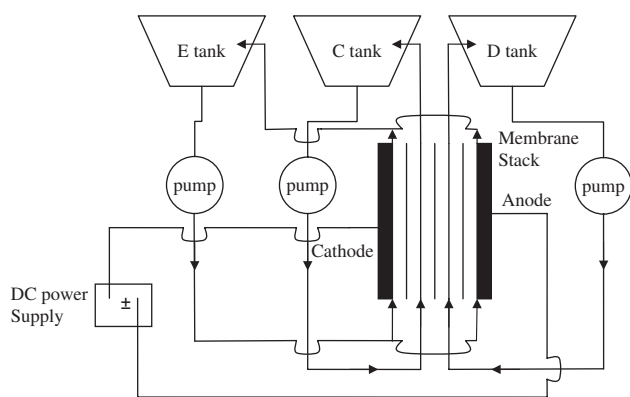
Fig. 1 – A schematic diagram of salt removal by electrodialysis.

in concentration tank (C tank) increased from 17 (initial) to approximately 36 (final) gL^{-1}. Conductivity of the feed (APWSH, D tank) was periodically checked with the goal of reducing it 10 fold. Initial and final conductivities of feed (APWSH) were 55 and 5 milli mhos cm^{-3}, respectively. At these conductivities, salt concentrations in APWSH were 21.72 and 1.98 gL^{-1}, respectively. Fig. 1 shows a schematic diagram of the electrodialysis apparatus used in these studies.

2.5. *Analyses*

Fermentation products (ABE, acetic acid, and butyric acid) were analyzed using gas chromatography (GC; 6890N; Agilent Technologies, Wilmington, DE, USA) as described previously [9,14,15]. Details of sugar and cell concentration measurements have been reported elsewhere [9]. ABE productivity was calculated as ABE produced in gL^{-1} divided by the fermentation time and is expressed as $gL^{-1}h^{-1}$. ABE yield was calculated as total ABE produced divided by the total sugar utilized. Salt concentration was measured with the help of a conductivity meter (Cole Parmer, Chicago, IL) using a standard curve plotted between NaCl concentration (gL^{-1}) and conductivity (milli mhos cm^{-3}). The results (fermentation) presented here are an average of two replications.

3. Results and discussion

In order to compare results of this investigation, a control batch fermentation was run with 60 gL^{-1} initial glucose concentration in the medium. The fermentation was run for 72 h and during this time period the culture produced 21.37 gL^{-1} total ABE resulting in a productivity of 0.30 gL^{-1} h^{-1}. During the fermentation, ABE yield of 0.36 was achieved [9]. Following this, butanol was produced using APWSH as the substrate. During 72 h of fermentation, 2.59 gL^{-1} ABE (acetone 0.98, butanol 1.26, and ethanol 0.35 gL^{-1}) was produced, which is low as compared with ABE produced in the control experiment suggesting that APWSH was toxic to the culture. Initial sugar level in the reactor was glucose 40.4, xylose 16.0, arabinose 2.3, and galactose 1.4 gL^{-1}, thus totaling 60.1 gL^{-1}. After fermentation, sugar level was glucose 35.8, xylose 14.0,

arabinose 0.8, and galactose 1.4 gL^{-1}. The total residual sugar level in the reactor was 52.0 gL^{-1}. In this run, ABE productivity and yield were 0.04 $gL^{-1}h^{-1}$ and 0.32, respectively. Poor fermentation performance in these experiments was likely due to presence of inhibitors such as salts and degradation chemicals in the hydrolysate. The initial salt concentration in the APWSH was 21.72 gL^{-1}.

One of the methods of relieving inhibition due to inhibitors is dilution of hydrolysate. Since fermentation of APWSH was toxic to the culture, the hydrolysate was diluted twice to reduce inhibitory level caused by toxic chemicals. In order to keep sugar level in the 60 gL^{-1} range, additional glucose solution was added to the reactor. Initial sugar (total 60.3 gL^{-1}) level in the reactor was glucose 40.6, xylose 15.7, arabinose 2.5, and galactose 1.5 gL^{-1}. Fermentation was complete in 72 h producing 12.80 gL^{-1} total ABE of which acetone, butanol, and ethanol was 2.97, 8.69, and 1.14 gL^{-1}, respectively (Fig. 2A). During the course of this fermentation, ABE productivity and yield were 0.18 $gL^{-1}h^{-1}$ and 0.37, respectively. Concentrations of various sugars during the fermentation are shown in Fig. 2B. At the end of fermentation, 26.1 gL^{-1} (glucose 13.7, xylose 9.3, arabinose 1.7, and galactose 1.4 gL^{-1}) total residual sugar was measured. The results of these experiments demonstrated that the hydrolysate contained toxic chemicals that inhibited fermentation.

Another solution to reduction of inhibition is removal of toxic chemicals prior to fermentation. Salts were considered to be likely major inhibiting chemicals in the hydrolysate (APWSH). To test this, ABE was produced using APWSH from which salts were removed by electrodialysis. In the fermentation reaction mixture, concentrations of various sugars were glucose 36.9, xylose 20.2, arabinose 1.8, and galactose 0.7 gL^{-1}. The total sugar level was 59.6 gL^{-1}. Fermentation of salt removed APWSH was rapid and produced 22.17 gL^{-1} total ABE (Fig. 3A). This ABE value is 3.7% higher than the control (glucose fermentation), which is within $\pm 5\%$ of error margins. The levels of individual products were acetone 9.34, butanol 12.33, and ethanol 0.5 gL^{-1}. As a result of reduction in inhibition, fermentation was complete in 40 h as opposed to 72 h in control experiments. This system resulted in ABE productivity and yield of 0.55 $gL^{-1}h^{-1}$ and 0.42, respectively. The concentrations of various residual sugars were glucose 0.5, xylose 5.5, arabinose 0, and galactose 0.6 gL^{-1} (total residual sugar 6.6 gL^{-1}) (Fig. 3B).

To further study if 20–25 gL^{-1} salt was inhibitory to *C. beijerinckii* P260, ABE fermentation studies with different NaCl concentrations in the medium were performed (Fig. 4). At a salt concentration of 25.0 gL^{-1}, no cell growth was observed. A salt concentration of 20.0 gL^{-1} resulted in production of 4.04 gL^{-1} total ABE suggesting that salts in APWSH to the concentration of 21.72 gL^{-1} were indeed the reasons behind the low production (2.59 gL^{-1}) of ABE in untreated APWSH cultures/fermentations. As shown in Fig. 4, NaCl above 2 gL^{-1} was inhibitory, both to cell growth and ABE fermentation. In our previous studies using *C. acetobutylicum* P262 and *C. beijerinckii* BA101, we demonstrated that salts were toxic to these cultures [3,4]. In those studies, commercial substrates such as whey permeate and soy molasses were used. It is likely that NaCl exerts osmotic pressure on the cell thus
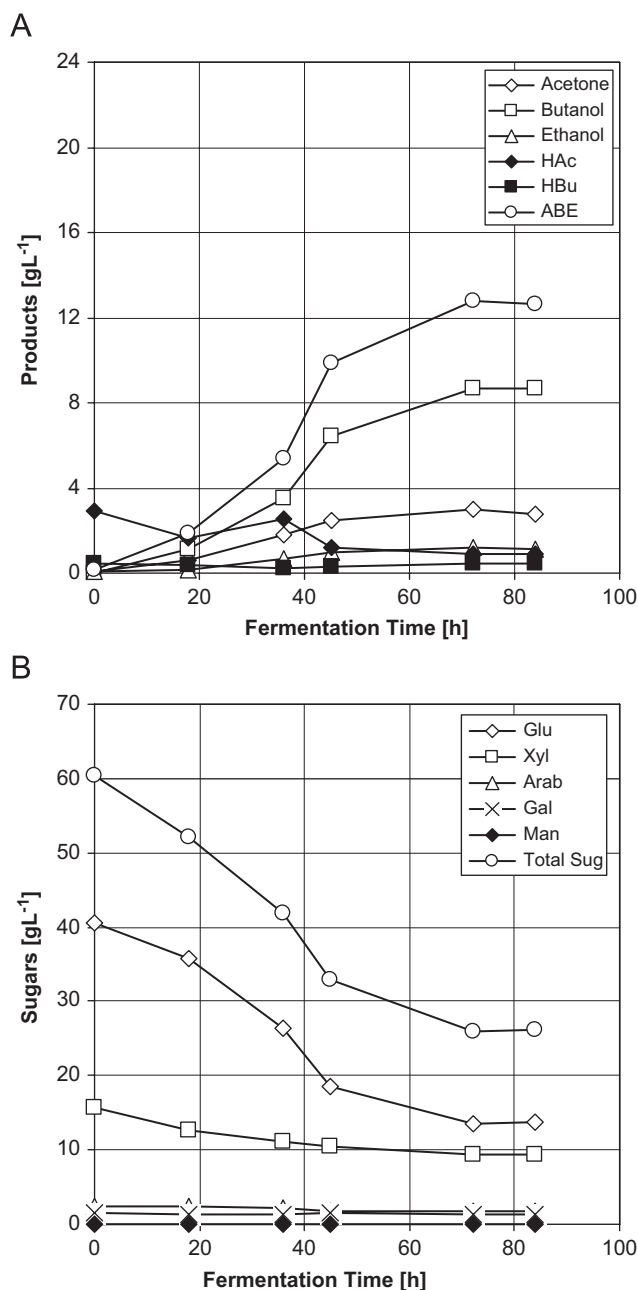
Fig. 2 – Production of ABE from diluted (twice) APWSH in a batch reactor using *C. beijerinckii* P260. (A) Products vs fermentation time; (B) sugars vs fermentation time.
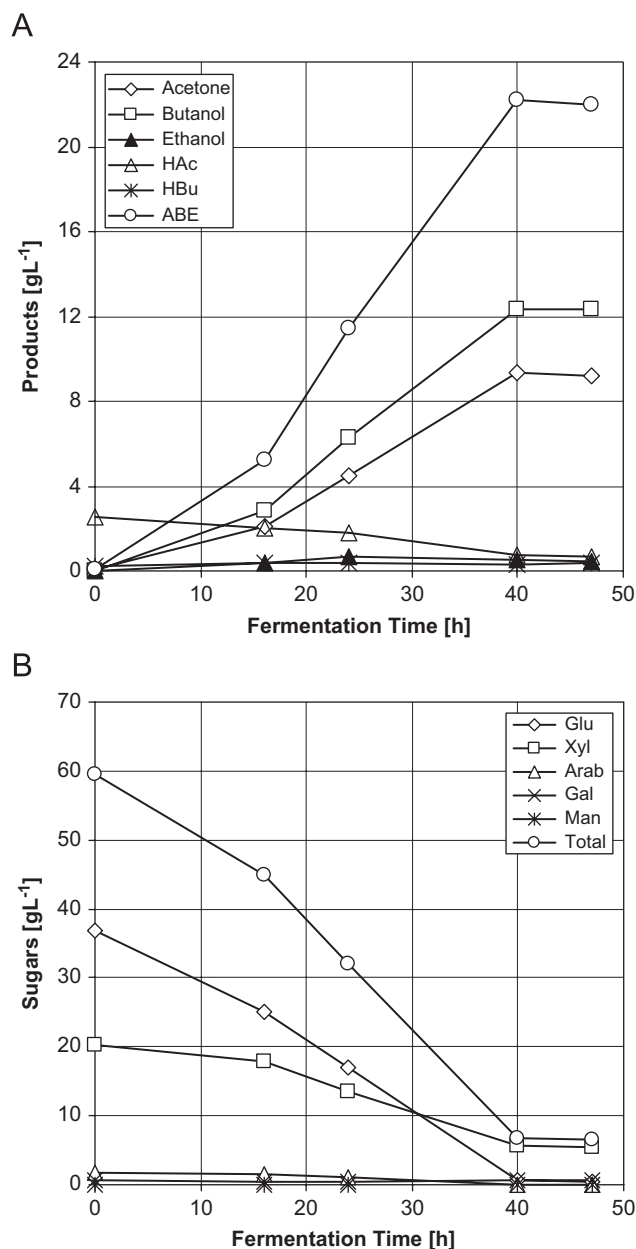

Fig. 3 – Production of ABE from APWSH after salt removal by electrodialysis in a batch reactor using *C. beijerinckii* P260. (A) Products vs fermentation time; (b) sugars vs fermentation time.

incapacitating it for growth and fermentation. However, there may be other mechanisms for growth and fermentation inhibition [5,6]. It is suggested that salts should be removed, perhaps by using electrodialysis, from commercial substrates before fermentation.

Fig. 5 shows a comparison of productivities and yield obtained during fermentation. Untreated APWSH was toxic to the culture and hence the culture could not produce more than $2.59 \, \mathrm{g \, L^{-1}}$ total ABE. The experiment run with diluted hydrolysate was less toxic to the culture and hence the culture produced $12.8 \, \mathrm{g \, L^{-1}}$ ABE. Reactor productivity was

improved from $0.04 \, \mathrm{g \, L^{-1} \, h^{-1}}$ using hydrolysate containing undiluted salts to $0.18 \, \mathrm{g \, L^{-1} \, h^{-1}}$ when the salt concentration was reduced to half as a result of dilution. ABE yield was also improved from 0.32 to 0.37. Removal of salts by electrodialysis further improved ABE production, productivity, and yield suggesting that electrodialysis was effective in removing inhibitory salts from the hydrolysate. Reactor productivity was $0.55 \, \mathrm{g \, L^{-1} \, h^{-1}}$ as compared with $0.30 \, \mathrm{g \, L^{-1} \, h^{-1}}$ in the control experiment. This productivity is 183% of the productivity achieved in the control experiment. ABE yield was also improved from 0.36 (glucose control) to 0.42 (after salts were removed). It is noteworthy that among the four runs, total
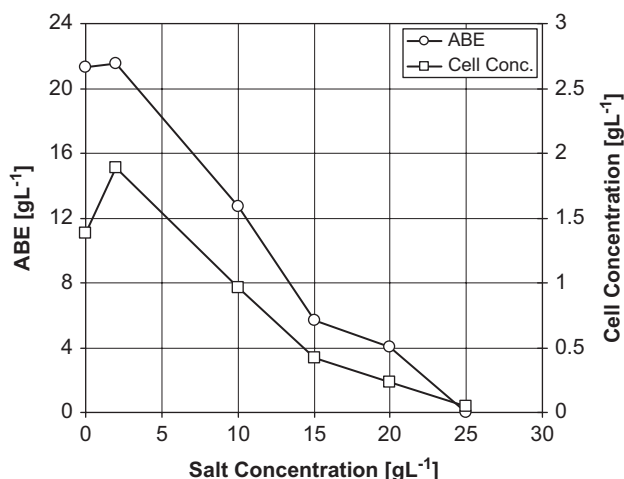
Fig. 4 – Production of ABE using _C. beijerinckii_ P260 from glucose containing various concentrations of NaCl.
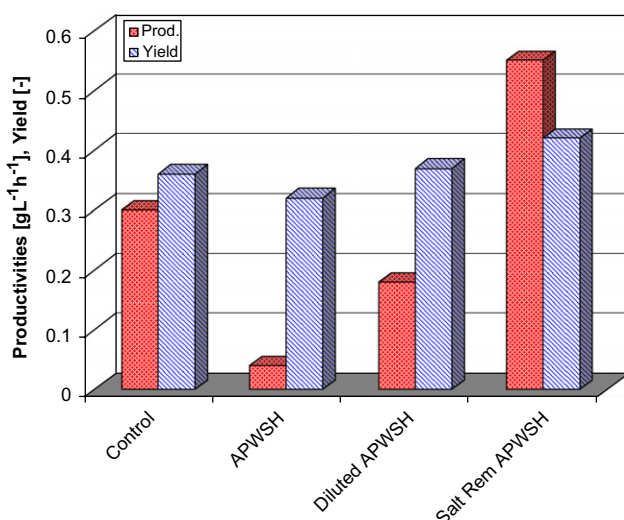

Fig. 5 – A comparison of ABE production kinetic parameters obtained in control, diluted APWSH, and salt-removed APWSH fermentations.

ABE production was highest ($22.17 \, gL^{-1}$) in the run where salts were removed. The total salt concentrations in APWSH before and after their removal were 21.72 and $1.98 \, gL^{-1}$, respectively.

In comparison, dilute sulfuric acid pretreated WSH was not toxic to the culture [8–10], while APWSH was found to be toxic, suggesting that toxicity is pretreatment specific. In our previous studies, we demonstrated that corn fiber hydrolysate (pretreated with dilute sulfuric acid) was toxic to _C. beijerinckii_ BA101 [16]. In contrast dilute sulfuric acid pretreated WSH stimulated butanol fermentation. It is likely that WSH contained furfural and hydroxymethyl furfural (HMF) that supported fermentation [2]. In our previous studies, it was demonstrated that presence of furfural and HMF in the medium stimulated butanol fermentation [2].

4.   Conclusions

It has been observed that the alkaline peroxide pretreatment technique generates salts that are inhibitory to _C. beijerinckii_ P260. However, fermentation was successful after these inhibitors (salts) were removed from the fermentation medium. A control fermentation resulted in the production of $21.37 \, gL^{-1}$ ABE while salt removed APWSH resulted in the production of $22.17 \, gL^{-1}$ ABE. In the two fermentations, reactor productivities were 0.30 and $0.55 \, gL^{-1} h^{-1}$, respectively. Fermentation of APWSH prior to removal of salts did not produce more than $2.59 \, gL^{-1}$ ABE. Fermentation studies with various added salt concentrations supported that NaCl was toxic to the culture. In these studies, we were successful removing fermentation inhibitor (salts) from the biomass hydrolysate medium. A comparison of use of different substrates (corn fiber, wheat straw) and different pretreatment techniques (dilute sulfuric acid, alkaline peroxide) suggests that generation of inhibitors is substrate and pretreatment specific. Further work on the use of various biomass substrates and different pretreatment techniques is being investigated in the author's (N. Qureshi) laboratory.

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